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Simultaneous Hyperaccumulation of Nickel, Manganese, and Calcium in *Alyssum* Leaf Trichomes

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We have developed commercially viable phytoremediation/phytomining technologies employing *Alyssum* Ni-hyperaccumulator species to quantitatively extract Ni from soils. The majority of Ni is stored either in *Alyssum* leaf epidermal cell vacuoles or in the basal portions only of the numerous stellate trichomes. Here, we report simultaneous and region-specific localization of high levels of Ni, Mn, and Ca within *Alyssum* trichomes as determined by scanning electron microscopy/energy-dispersive X-ray analysis (SEM/EDX). Plants were grown in high Ni soil, achieving up to 48 400 μg g^{-1} Ni in total leaf concentration; however, Ca and Mn were not enriched in the experimental soils. The region-specific localization of hyperaccumulated Ca, Mn, and Ni occurred in three soil types, five *Alyssum* species/ecotypes, and over a wide range of soil Ni concentrations. The metal concentration in the trichome basal compartment was ∼15−20% dry weight, the highest ever reported for healthy vascular plant tissue.

Introduction

More than 400 plant species are known to naturally accumulate high levels of metals such as Cd, Cu, Co, Mn, Ni, and Zn. Hyperaccumulation is defined as accumulation of >1000 μg g^{-1} in plant dry material for Cd, Ni, and Co, and >10 000 μg g^{-1} for essential trace elements such as Mn and Zn which are required in larger amounts to support normal metabolism (*1*). The genus *Alyssum* (Brassicaceae) contains the greatest number of reported Ni hyperaccumulators (48), many of which can achieve 3% dry weight Ni in leaf biomass (*2*). Our research consortium has developed commercially feasible phytoremediation and phytomining technologies that can potentially clean up Ni-contaminated soils (*3*−*5*). The technology employs the Ni-hyperaccumulating species *Alyssum murale* and *A. corsicum* to quantitatively extract Ni from a range of soil types. These species are endemic to serpentine (ultramafic-derived) soils throughout Mediterranean Europe, but unlike many serpentine-endemic species, they grow prolifically and hyperaccumulate Ni in other soil types such as limestone soils, organic soils, and loam (*4*−*6*).

Successful development of hyperaccumulator species for large-scale phytoremediation/phytomining requires knowledge of Ni localization patterns for each genus/species/ecotype of interest, and for a variety of realistic growth conditions. The Ni localization patterns have been determined for 10 *Alyssum* Ni hyperaccumulator species/ecotypes (*6*−*11*). Nickel is mainly stored in the leaves and is particularly concentrated in epidermal cell vacuoles. There is a positive correlation of sulfur (S) and Ni, indicating that SO_4^{2-} may be a counterion for Ni^{2+} within the vacuoles (*6*, *9*).

The upper and lower leaf surfaces of *Alyssum sp.* are covered with an overlapping network of branchlike, stalked bifurcate or stellate trichomes (*10*, *12*, *13*). The 10 *Alyssum* Ni hyperaccumulator species/ecotypes mentioned above have stellate trichomes with 8−14 elongate rays, some of which bifurcate. The upper sides of the rays are covered with hemispherical nodules, but the underside is smooth (Figure 1). Trichomes are attached to the epidermis with an ∼20 μm smooth cylindrical pedicle that has a broad fan-shaped compartment at its base. We found previously for *A. murale* that the trichome basal compartment, trichome pedicle, and the epidermal cells adjacent to the trichome basal compartment strongly concentrate Ni, but there was no appreciable Ni in the rays or nodules (*6*). Here, we report simultaneous and region-specific localization of high levels of Ni, Mn, and Ca within *Alyssum* trichomes.

Materials and Methods

Horticulture. *Alyssum murale* "Kotodesh", *A. murale* "AJ9", *A. corsicum*, *A. fallcinium*, and *A. pterocarpum* were grown from seed in a greenhouse at USDA Beltsville. Twenty-one days later, "Kotodesh" seedlings were transplanted to 250 g pots containing Promix potting soil with an increasing series of $NiSO_4 \cdot 6H_2O$ addition (0, 5, 10, 20, 40, 80 mmol Ni kg^{-1}). $NiSO_4$ is a standard salt for Ni soil enrichment. Carbonates (half $CaCO_3$/half $MgCO_3$) were added to each pot at amounts equimolar with $NiSO_4$. *Alyssum murale* "Kotodesh", *A. murale* "AJ9", *A. corsicum*, *A. fallcinium*, and *A. pterocarpum* were also transplanted into calcareous mineral soil associated with nickel mining tailings from Inco Ltd. operations at Port Colborne, Ontario (Welland soil, Typic Epiaquoll; Canadian classification, Terric Mesisol with 30 g kg^{-1} $CaCO_3$ added). The Inco soil typically yields 1% Ni in *Alyssum* whole shoots and 1.7% in leaves and would be phytotoxic to normal plants (*14*). For control plants, all five *Alyssum* species were transplanted into Promix only.

Initial results showed a significant correlation of Ni and S, especially in epidermal cell vacuoles. Because the possibility existed that additional S was provided by $NiSO_4$ or $MgSO_4$, we started a second group of plants in soils with no added S. *Alyssum murale* "Kotodesh" was grown from seed and transplanted after 21 days into 250 g pots containing Promix with an increasing series of $NiC_4H_6O_4 \cdot 5H_2O$ addition (0, 5, 10, 30, 60, 90 mmol Ni kg^{-1}), and equimolar carbonates as above. *Alyssum murale* "Kotodesh", *A. murale* "AJ9", and *A. pterocarpum* seedlings were also transplanted into natural Brockman variant serpentine soil from Josephine Co., Oregon (Typic Xerochrepts), with 10 wt % Promix added to improve drainage.

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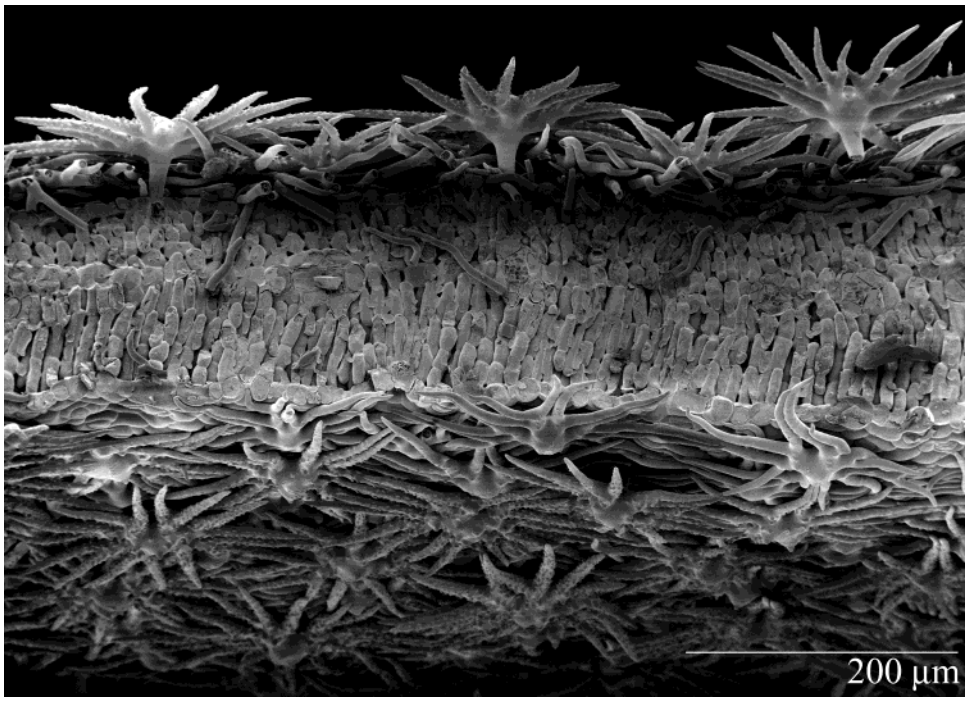

FIGURE 1. SEM image (2.0 kV) of a frozen-hydrated *Alyssum murale* "Kotodesh" leaf cross section. Trichomes project from the upper (top of figure) and lower epidermis; note the cylindrical pedicles and attachment points visible on the upper epidermis. For this image, a fresh leaf from a plant grown in natural serpentine soil was bulk frozen and complement fractured in liquid nitrogen, and it remained frozen throughout the analysis.

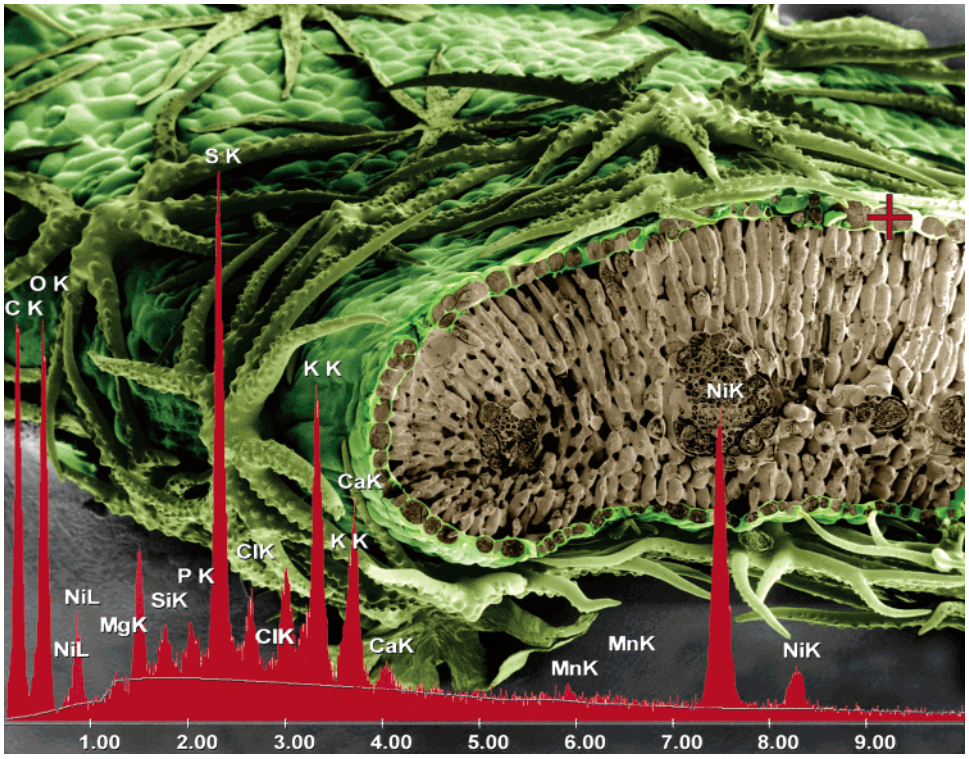

FIGURE 2. SEM image (2.0 kV) of a frozen-hydrated leaf cross section from the Ni hyperaccumulator *Alyssum murale* "Kotodesh". Unicellular trichomes cover the upper, lower, and lateral epidermis. The plant was grown in Promix with 40 mmol kg^{-1} Ni added. Overlaid is the X-ray spectrum (20 kV) from an upper epidermal cell vacuole, marked with red cross-hairs. Elemental Kα and Kβ signals are shown; note also Ni Lα and Lβ signals just below 1.0 kV. The *Y*-axis is peak/background X-ray counts in 100 live seconds. Five *Alyssum* hyperaccumulator species/ecotypes examined had leaf epidermal vacuoles containing high concentrations of Ni associated with elevated S.

The experiments were conducted in the greenhouse under controlled temperature and light conditions and ambient humidity. The photoperiod was 15/9 day/night. High-intensity sodium and incandescent lights supplying 400 μmol m^{-2} s^{-1} supplemented sunlight if necessary. The day temperature was 24 °C with cooling initiated at 27 °C. The night temperature was 18 °C with cooling initiated at 21 °C. Plants were grown in freely drained plastic pots with saucers to

prevent loss of leachate and were watered with deionized water. The plants received standard fertilization (Miracle Grow) once per month, and $MgSO_4$ (1.5 g L^{-1}) bimonthly for the $NiSO_4$ series, Inco, and control plants. The $NiC_4H_6O_4$ plants received 1.5 g of supplemental $MgCO_3$ bimonthly, and the serpentine soil plants did not receive supplemental Mg.

Total Metals Analysis. Two-centimeter lengths of apical stem tip from three locations on each plant were harvested for total metals analysis. Intermediate age branches were sampled; that is, neither the youngest nor the oldest leaves were utilized. Ca, Cu, Mg, Mn, Ni, and Zn were determined by inductively coupled plasma atomic emission spectrometry (Perkin-Elmer Optima 4300 DV) using 40 mg L^{-1} yttrium as an internal standard. Detailed methods are given in ref *6*. For quality control, reagent blanks and an in-house *Alyssum* standard were included. Each sample was analyzed at least three times. Duplicates differing by more than 10% are automatically excluded from the dataset, and the sample was subsequently reanalyzed. We were primarily interested in Ni concentrations, but we also analyzed for selected essential nutrient trace elements present at relatively high concentrations to better understand the plants' response to increased soil Ni.

Scanning Electron and X-ray Spectrometry. For microanalysis, bulk leaf samples were frozen in liquid nitrogen and fractured to yield complement cross sections (*6*). One-half of each complement remained frozen-hydrated and was etched and imaged with cryogenic SEM at 2.0 kV. The other half was freeze-dried and examined with SEM/EDX at 20 kV.

Results and Discussion

Figure 2 illustrates the botanical detail and precise determination of metal localization that our methods achieve. The Ni levels that a healthy, thriving *Alyssum* hyperaccumulator species can attain in the epidermis are extraordinary; under normal circumstances, any transition-metal X-ray signal is undetectable in plant material with SEM-EDX methods.

For *A. murale* "Kotodesh", we have determined that the trichome basal compartment, pedicle, and rays consist of a single large cell (Figure 3). Trichome physiology is nearly identical in four other species/ecotypes we have investigated. Figure 4 summarizes trichome metal localization data for all

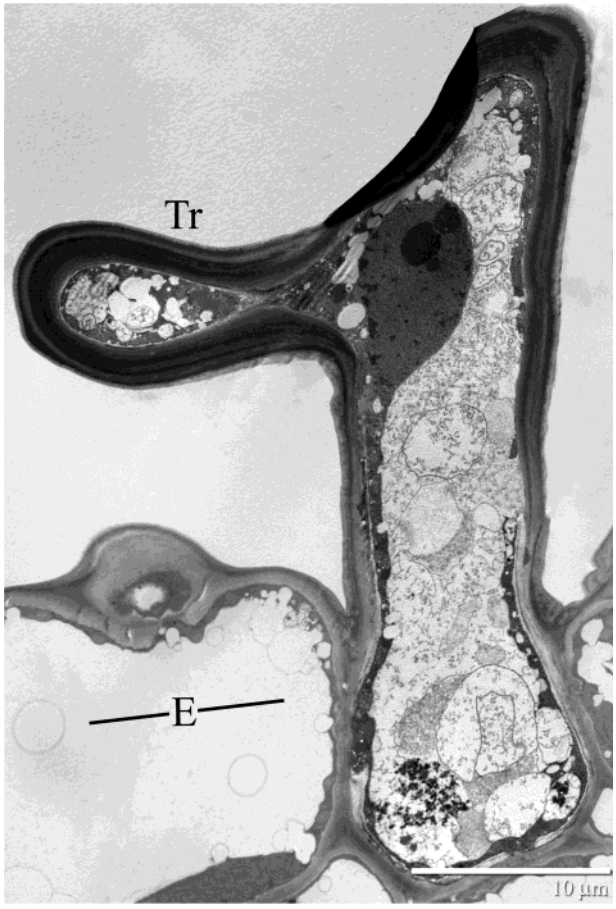

FIGURE 3. Transmission electron micrograph (75 kV) of a leaf thin cross section taken from the plant in Figure 2. The upper epidermal surface lies across the lower third of the image (—E—), with a trichome pedicle projecting upward, perpendicular to the epidermal surface. A trichome ray (Tr) extends from the pedicle, roughly parallel to the epidermal surface. The entire trichome including the basal compartment consists of a single cell. Fresh leaf was fixed in 3% gluteraldehyde and 0.2 M Na cacodylate and was mounted in Spurr resin.

TABLE 1. Selected Element Concentrations in *Alyssum* Leaves[a]

sample	Ni	Ca	Cu (μg g^{-1})	Mg (dry wt)	Mn	Zn
A. murale "Kotodesh" 00	5.20	20 700	7.23	2740	154	145
A. murale "Kotodesh" Inco a	3040	43 700	25.4	3220	125	1480
A. murale "Kotodesh" Inco b	1670	42 800	14.2	2500	122	139
A. murale "Kotodesh" OR	8038	32 400	9.00	3540	801	255
A. murale "AJ9" 00	5.00	48 400	8.58	4690	295	55.1
A. murale "AJ9" Inco a	3350	29 700	7.69	5005	133	40.4
A. murale "AJ9" Inco b	3520	29 500	11.4	4130	155	44.4
A. corsicum 00	9.72	31 300	4.65	6050	195	178
A. corsicum Inco a	10 360	40 000	10.6	5030	89.9	177
A. corsicum Inco b	7430	38 000	15.5	4810	110	171
A. corsicum OR	7600	32 100	7.20	6400	289	217
A. pterocarpum 00	32.0	22 300	6.87	11 500	139	146
A. pterocarpum Inco a	7130	24 900	12.8	5063	38.2	56.9
A. pterocarpum Inco b	8670	29 100	16.3	6190	44.5	77.3
A. pterocarpum OR	11 600	24 400	9.50	7440	215	169
A. fallcinium 00	111	35 800	13.3	12 000	70.6	324
A. fallcinium Inco a	5300	36 900	24.3	3190	88.3	94.5
A. fallcinium Inco b	6600	39 400	14.7	4610	114	84.6
Alyssum std. 1	15 700	12 200	0.02	2080	23.4	60.2
Alyssum std. 2	17 800	12 600	0.82	2122	20.9	63.3
Alyssum std. 3	17 100	11 800	0.60	2060	24.0	65.0

[a] Letters a and b represent analyses of duplicate plants/treatments. Control (Promix only) plants designated by 00. Inco samples are plants grown in contaminated mineral soil from a Ni refinery area in Ontario. OR samples are plants grown in serpentine soil from the Josephine Co., OR. Values are the mean of three analyses agreeing by better than 90% and rounded to three figures; the standard error is insignificant. Element concentrations for the $NiSO_4$ and $NiC_4H_6O_4$ series plants are given in ref *6*.

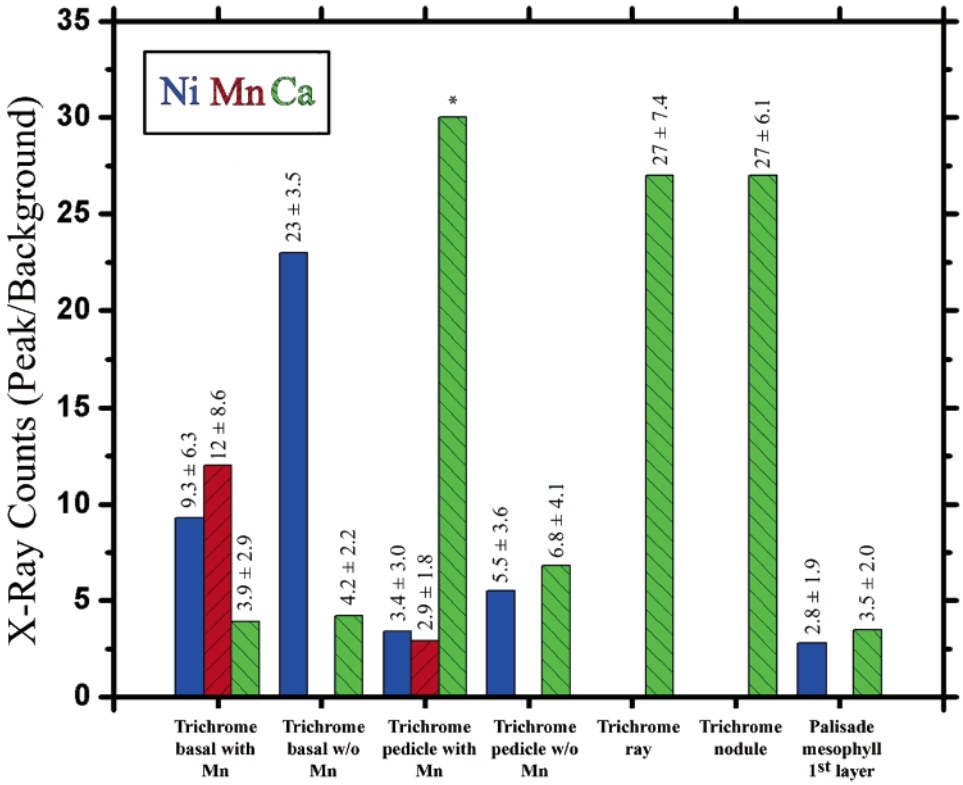

FIGURE 4. Mean Ni, Ca, and Mn peak/background Kα X-ray counts (20 kV; 100 ls) in various areas of trichomes on the leaves of five *Alyssum* Ni hyperaccumulator species/ecotypes grown in various Ni-rich soils. Palisade mesophyll cell data are given as a comparison. Ni (shaded), Mn (SW to NE cross-hatch), and Ca (NW to SE cross-hatch) left to right. Region-specific localization of metals within the trichome is independent of soil type or soil Ni concentration. Trichome basal = trichome basal compartment. Number of samples analyzed for respective entries, left to right 9, 4, 3, 10, 7, 8, 21. Error values indicate range of element concentration variation within the plant tissue type and among species, not analytical error (<3%). The asterisked entry has a range too large to utilize standard error because Ca counts were highly variable depending on the exact position on the pedicle that was analyzed.

five *Alyssum* in different soil types and at various Ni soil levels. It includes data from $NiSO_4$, $NiC_4H_6O_4$, Inco, and serpentine soil experiments. There was no enrichment of Mn in any experimental soil, and Ca was not added beyond the initial $CaCO_3$ application. Note that Figure 4 is an overview of previously unreported *Alyssum* trichome behavior and is not intended to statistically define metal concentration ranges in various tissue types/locations. An interesting observation is that metal hyperaccumulation patterns in the trichomes are consistent despite the variations in soil type, species/ecotype, and Ni soil level.

Calcium was strongly concentrated in trichome rays and nodules. Trichome nodule analyses detected mainly C, O, and Ca, consistent with the presence of calcium carbonate or oxalate crystallites (*6*, *8*−*10*, *15*). Calcium levels decreased and Ni increased as one moved down the pedicle away from the rays and toward the epidermis proper. Calcium levels in the pedicle could vary from near detection limits to over 30 peak/background counts depending on the exact position on the pedicle that was analyzed. On occasion, Mn was strongly concentrated along with Ni in trichome pedicles and especially basal compartments.

Figure 5 shows that Ca, Ni, and Mn are all hyperaccumulated, yet localized in specific areas of the trichome base and pedicle. At locations where both Mn and Ni were detected, Ni X-ray counts were relatively reduced as compared to trichomes where Mn was not present. Although Ca is not generally considered a hyperaccumulated metal (*1*, *2*), the Ca concentration can exceed 30% dry weight in trichome nodules. Therefore, for purposes of this discussion, we consider it to be hyperaccumulated along with Ni and Mn because the Ca concentration exceeds epidermal cell physiological levels by 2 orders of magnitude.

The metal concentration at any pixel in Figure 5 is conservatively 15−20% dry weight, which is the highest ever reported for healthy vascular plant tissue (excluding crystallites and phytoliths). The Ni-hyperaccumulator *Sebertia acuminata*, a tropical serpentine-endemic tree, is the only other plant reported to contain such elevated metal levels. *Sebertia acuminata* was found to have 18.5% and 25.8% dry weight Ni in two separate analyses of its latex; however, leaf and seed tissue contained 1% and 0.5% Ni, respectively (*16*). Because the SEM-EDX analysis is semiquantitative, concentration values can only be estimated by applying the ZAF calculation to the peak/background counts measured at selected locations with favorable geometry (*6*, *9*, *17*). Eleven individual locations were point-analyzed to correlate bitmap signal intensities with metal concentrations.

Previously, there was no consensus as to whether trichomes in various species are a storage location for hyperaccumulated metals. Psaras et al. (*10*) found no Ni in trichomes, whereas proton microprobe analysis of freeze-dried *A. lesbiacum* leaf cross sections found that Ni was concentrated in trichomes (*11*). Marmiroli et al. (*8*) found one case where an *A. lesbiacum* trichome had broken off and was inverted. An X-ray bit map showed elevated Ni counts in the hole where the pedicle was attached but not on the trichome outer surface. Identical results were observed for *A. lesbiacum*; Ni was absent from the trichome itself, but there was a suggestion (observed by staining) that Ni was concentrated at the pedicle attachment point (*9*).

In the Zn hyperaccumulator *Arabidopsis halleri*, Zn and Cd were consistently concentrated at the base of leaf trichomes, but not distributed throughout the trichome (*14*, *18*). Blamey et al. (*19*) reported that Mn was concentrated at the base of leaf trichomes in sunflower (*Helianthus annuus*),

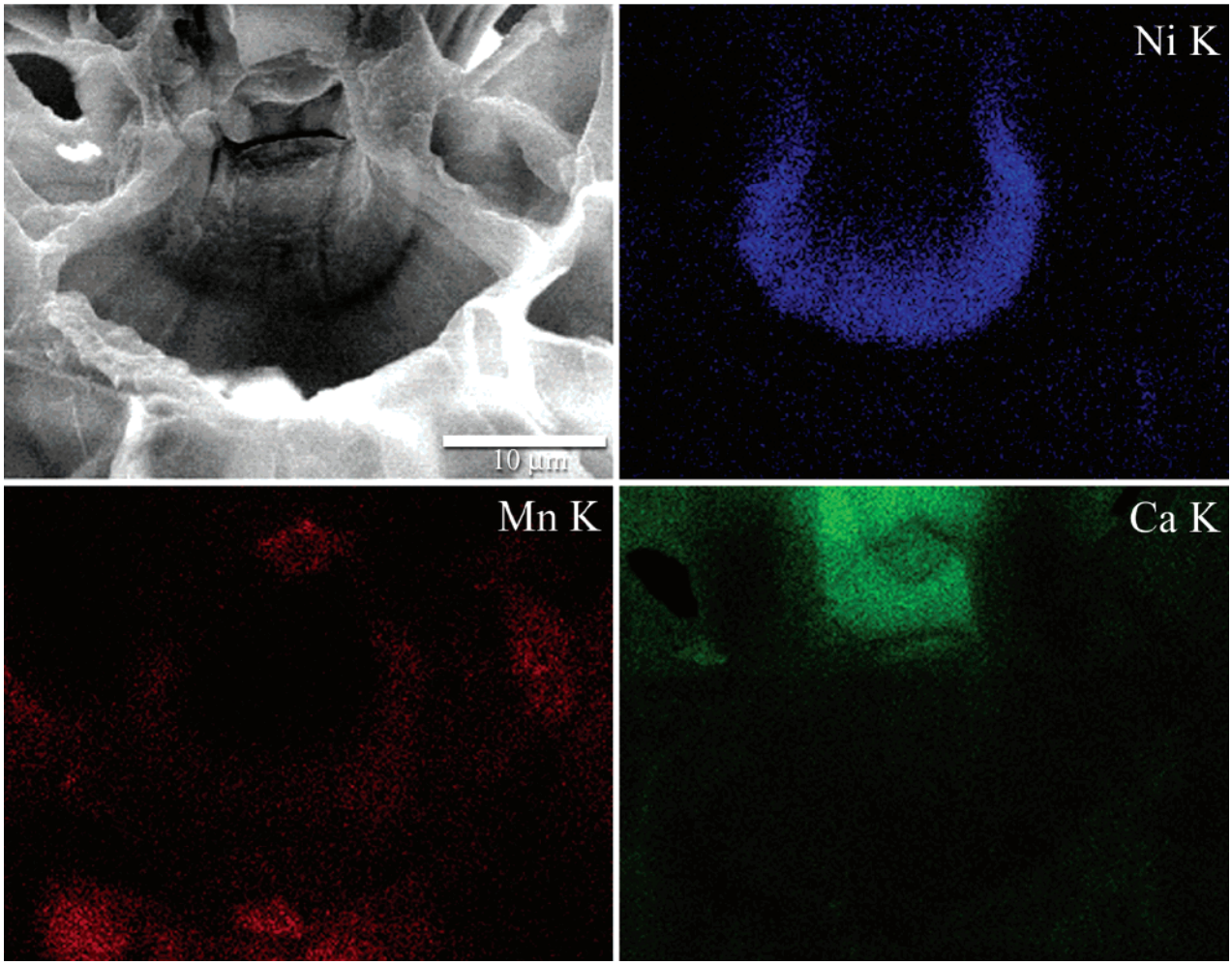

FIGURE 5. SEM-EDX image and Kα X-ray bitmaps (20 kV, 1.0 h counting) of a trichome basal compartment. Nickel, Mn, and Ca are simultaneously hyperaccumulated, yet localized in specific areas of the trichome basal compartment and pedicle. Only Ca is hyperaccumulated further up in the trichome rays and nodules.

possibly as a response to potential toxicity. Manganese distribution was strongly heterogeneous in the *H. annuus* leaf. Heterogeneity of Mn distribution is observed in many plants, with characteristic high Mn "spots" on leaves as opposed to uniform tissue Mn elevation (*20*). In Promix with Ni salt addition, whole leaf Ni levels increased with increasing Ni addition, but Mn and Ni concentrations were not correlated (*6*). Our X-ray data and whole-leaf metal analyses (Table 1) are consistent with heterogeneous Mn distribution in *Alyssum* that may be controlled by trichomes. For *Alyssum* grown in Inco and serpentine soils, whole leaf Ni concentrations were greatly increased as compared to control plants, but there was no corresponding Mn elevation. A full analysis of possible subtle trace element correlations will require more samples and the determination of soil Mn availability.

Recently, Epimashko et al. (*21*) observed two functionally different vacuoles within a single mesophyll cell in ice plant (*Mesembryanthemum crystallinum*), a halophyte. Under salt stress, large amounts of NaCl were stored in one type of vacuole, while a second type operated for malate cycling. This means that determination of Na cellular concentrations by bulk sap sampling may lead to erroneous results. *Alyssum* Ni-hyperaccumulator trichome basal compartments (and perhaps all epidermal cells) have similar subcellular complexity. Figure 5 indicates that Ni handling and storage is physically separate from Ca and Mn handling/storage and could also be metabolically distinct.

Phytoremediation with lowest-cost means is presently known to return metal-contaminated soil to productive use (*4*, *5*, *22*, *23*). While many hyperaccumulators are known, few are considered viable for large-scale quantitative metal extraction. *A. murale* and *A. corsicum* are exceptional in that they have real potential to be grown as agricultural crops (*4*, *5*). They (a) have high biomass, which can be further increased with standard crop and soil management practices; (b) have an upright branching habit which is amenable to mechanical harvesting; (c) are adapted to temperate climates; and (d) accumulate higher Ni concentrations than exist in the soil without suffering symptoms of toxicity. Additionally, we have found that *Alyssum* trichomes are specialized cells capable of handling and compartmentalizing very high concentrations of metals, and this behavior may be generalized to trichomes of other hyperaccumulator taxa. A greater understanding of trichome physiology and cellular metabolism is a necessary aspect of further phytoremediation/phytomining research. It is possible, for example, that an ability to utilize trichomes for metal sequestration is one reason a particular hyperaccumulator can achieve high biomass on serpentine or other nonfertile metal-contaminated soils.

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